Electrochemical sensors for detection of tetracycline antibiotics

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Abstract

An electrochemical immunosensor based on screen-printed carbon working electrode with onboard carbon counter and silver–silver chloride pseudo-reference electrode for tetracycline antibiotic detection is described in this paper. A direct competitive enzyme-linked immunosorbent assay (ELISA) format was first developed and optimized on the surface of a carbon screen-printed working electrode by immobilizing the capture antibody (polyclonal) using electro-deposition of gold nanoparticles conjugated with polyclonal anti- tetracycline antibodies. The detection reagent, tetracycline-Horseradish Peroxidase (HRP) conjugate was used as an enzyme label. Electrochemical detection was then carried out using 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) /H_2O_2 as the enzyme mediator /substrate system and conducted using chronoamperometry at 300mV vs. onboard screen-printed Ag-AgCl pseudo-reference electrode. Tetracycline detection of 10 ppb was achieved. The optimal sensor configuration was then compared to a commercial ELISA kit method for analyzing meat samples. The sensor was also capable to detect four derivatives of tetracycline tested such as Oxytetracycline, Doxycline, Chlortetracycline and Demeclocycline. The sensor showed good selectivity for tetracycline with higher recovery percentage (70 - 95%) in the presence of other antibiotics in the sample. It also showed very high sensitivity using the amplification strategy applied with the use of gold nano-particles. This makes the developed sensor a potential tool for a rapid, simple and sensitive detection of tetracycline residues in poultry products.

Keywords: Gold nanoparticles, immunosensor, screen-printed gold electrode, tetracycline, rapid detection, food monitoring.

Introduction

Antibiotics are used in animal farming to prevent and treat diseases and to promote the growth of animals. Antibiotic growth promoters improve production by cutting animal finishing time, reducing the feed conversion rate, decreasing the incidence of animal mortality and by improving the animal's overall condition. Many countries have been moving towards a complete ban on antibiotic growth promoters in livestock production for some time. The use of antibiotics for agricultural purposes has created concerns about antimicrobial resistance, which is believed to cause antibiotics to become ineffective in the treatment of diseases due to the emergence of antibiotic resistant infectious agents. Consequently, despite their proven efficacy, the use of antibiotics as growth promoters is no longer encouraged. According to World Health Organisation (WHO), more than half of global production of antibiotics is used on farm animals. In the last 30 years the use of penicillin-type drugs in farm animals has

increased by 600%. Therefore, many countries are increasingly shifting towards banning and monitoring the use of antibiotics in animal farming industry.

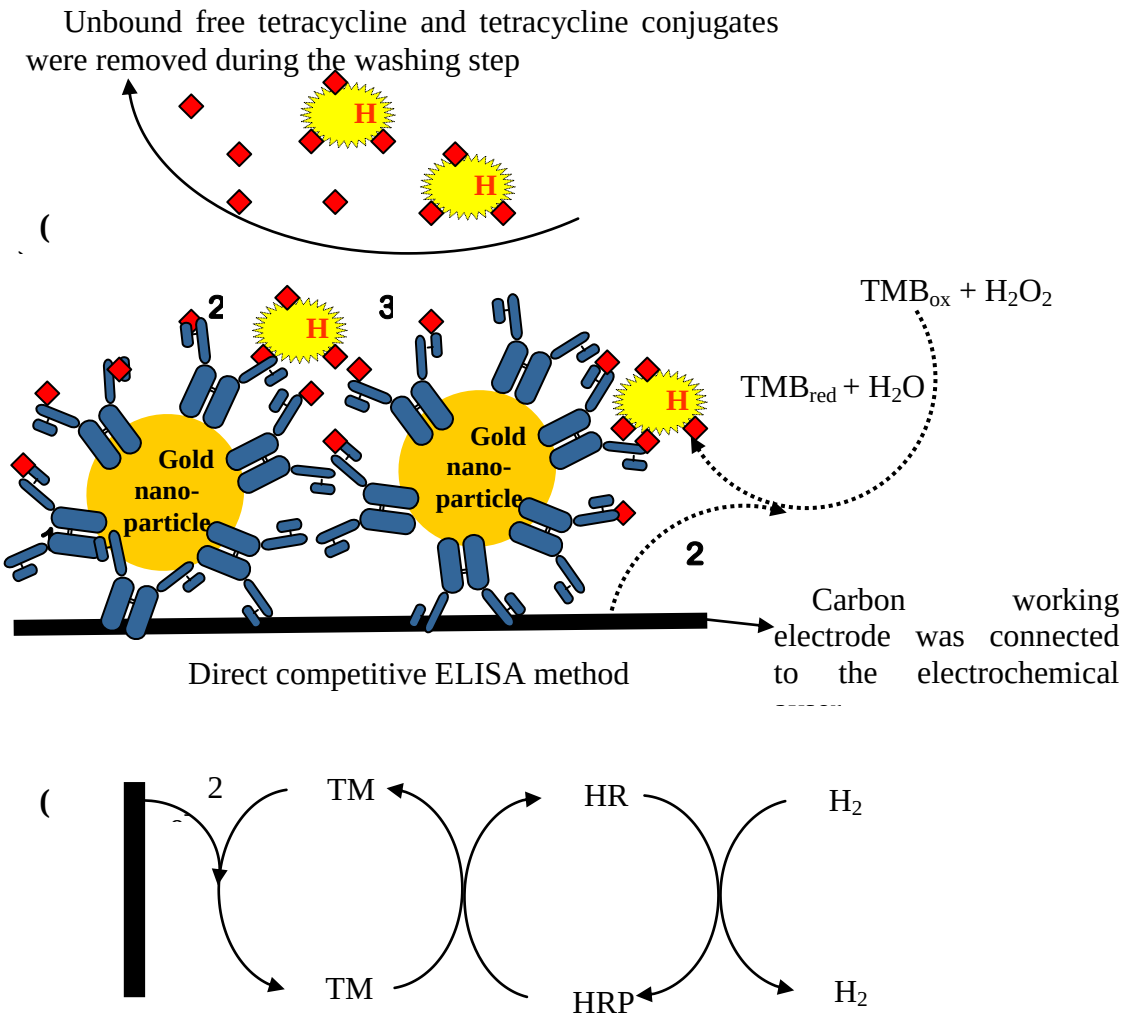

Figure 1. (a) Electrochemical immunosensor format used for tetracycline detection: **(1)** the specific antibody against tetracycline which was physically adsorbed on gold surface of gold nano-particle and was electro-deposited on carbon working electrode, **(2)** tetracycline antibiotic were captured by the antibody and, **(3)** tetracycline conjugated with HRP acting as the detector which gave the signal when TMB-H_2O_2 substrate was applied to the electrode surface. (b) Complex TMB/HRP/H_2O_2 enzyme reaction showing indirect electron transfer for TMB oxidation/reduction on the carbon working electrode surface for the formation of reduction current (Salam and Tothill, 2009).

At present many of the currently used methods for tetracycline detection are time consuming (tedious extraction procedure) and high instrumentation cost. Therefore, rapid detection of tetracycline antibiotic is a key to the prevention of problems related to health and safety. Rapid detection of antibiotics, pathogens, spoilage microorganisms and other microbial contaminants in foods is critical to ensure food safety and quality (Tothill, 2011; Lazcka *et al*., 2007). Currently, biosensor devices can offer a very attractive alternative technology for contaminant detection since they can be rapid, sensitive and simple to perform (Alocilja and Radke, 2003) as well as can provide real-time and on-site analysis (Tothill, 2011). Real-time detection of chemical contaminants is important since it provides immediate interactive information regarding the sample being tested and enables food manufacturers to take corrective measures before the product is released for consumption. Electrochemical immunosensors are an example of biosensor technology which is attracting great attention as tools suitable for rapid and sensitive analysis with wide application in the medical, food and environmental sectors (Heurich *et al*., 2011; Liang *et al*., 2005; Wilson and Rauh, 2004).

Recent reviews on nanotechnology provide a new prospect for the use of nano material labels for signal amplification in electrochemical immunosensors (Kerman *et al*., 2008). In this work an electrochemical immunosensor was developed where the capture antibody (polyclonal antibody raised against tetracycline antibiotic) was immobilized on the working carbon electrode surface. The detection enzyme conjugates (tetracycline-HRP) will form the competitive assay on the sensor surface. The current measurement of the enzyme-substrate reaction was then conducted using an electrochemical system comprising of an electron transfer mediator, 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) with H_2O_2 as the substrate system (Volpe *et al*., 1998). This resulted in current amplification since several antibody molecules were attached to each particle acting as signal enhancement enabling lower detection limit for tetracycline in chicken samples (Figure 1). This paper focuses on enhancing the signal of electrochemical immunosensors by using gold nano-particles in the detection reagent for tetracycline antibiotic screening.

Materials and Methods

Reagents

Tetracycline R-Biopharm ELISA Kit was purchased from R-Biopharm, Germany. Rabbit anti-mouse IgG conjugate with Horseradish Peroxidase (HRP) were purchased from Abcam Ltd., UK. Polyclonal antibody raised against tetracycline was developed in the Animal Complex, Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute, Serdang, Selangor, Malaysia. Polypyrrole, Phosphate buffer saline tablets, 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution, potassium chloride (KCl), tetracycline-HRP conjugate and potassium ferrocyanide ($K_4Fe(CN)_63H_2O$) were purchased from Sigma, Dorset, UK. Double distilled deinoised water was used for cleaning of the glassware and dissolution of the compounds.

Buffers and solutions

Phosphate buffered saline (PBS), comprising of 0.13mM NaH_2PO_4, 0.5mM Na_2HPO_4 and 0.51mM NaCl, pH 7.4 was prepared by dissolving five buffer tablets in 1L distilled- deionised water. For the analysis, ready-made TMB solution was

used. According to the manufacturer (Europa Bioproducts Ltd., Ely, UK), the TMB solution is stable at room temperature and is not sensitive to normal laboratory light. It is optimized with respect to TMB and hydrogen peroxide concentrations and yields a linear response with the concentrations of HRP usually employed in immunological assays. It also contains stabilizers.

Screen-printed carbon electrode fabrication and electrochemical assays

Screen-printed carbon electrodes (SPCE) consisting of carbon working electrode, carbon counter electrode and silver–silver chloride pseudo-reference electrode were fabricated by Screen Print Technology Sdn Bhd. Sungai Petani, Malaysia. The SPCE used in this work consisted of a carbon working electrode with a 5 mm diameter giving a 19.6 mm^2 planar area. All electrodes were then tested using a multimeter before use. The sensor edge connector was purchased from DropSense Ltd. UK through Metrohm Sdn Bhd, its product distributor in Malaysia.

Electrochemical measurements were carried out by placing a 50 µL solution onto the electrode, covering the 3-electrode area. Each measurement was carried out in triplicates using a new strip in a non-deaerated and unstirred solution. Measurements were performed using the Autolab Type II (Eco Chemie, The Netherlands) with NOVA 1.6 software. Cyclic voltammetric measurements were carried out by scanning at 50 mV s^{-1} between -1.0 and 1.0 V relative to Ag/AgCl reference electrode. Stock solutions of 50 mM potassium ferrocyanide were prepared in 0.1 M KCl. For the selection of optimal potential for TMB-H_2O_2-HRP system, chrono-amperometry was conducted similar to that reported previously (Salam and Tothill, 2009).

Preparation of conjugated gold nano-particles with antibody and Horseradish Peroxidase enzyme

Gold nano-particles with average diameter of approximately 20 nm were purchased from BBInternational, Cardiff, UK. The colloidal gold solution was stored in a dark bottle at 4 ^{o} C and used directly without any pre-treatment. The particle size of colloidal gold was ~ 20 ± 3.2 nm and the number of particles is ~ 7 x 10^{11} particles per mL. The UV-vis spectrometer showed an absorption peak at 525 nm. The antibody-colloid gold conjugate was prepared according to the procedure described by Chen *et al*. (2007). The antibody-colloidal gold conjugate was prepared by adding 100 µL of anti-tetracycline antibody (1.0 mg mL^{-1}) to 1.0 mL of pH-adjusted colloidal gold solution (pH 9.0), followed by slow shaking for one hour at room temperature. This allowed the antibody to adsorb onto the gold nanoparticles through a combination of ionic and hydrophobic interactions. Then, 100 µL of 10% BSA solution was added to the gold-antibody mixture and left for further 30 minutes at room temperature. The mixture was then centrifuged at 9,000 rpm for 30 minutes. The supernatant solution was then discarded and the soft sediment of immuno-gold was dissolved in 100 µL of 1% BSA solution and stored at 4 ^{o}C until used. The BSA was used to stabilize the immuno-gold colloid and minimize the non-specific adsorption during the assays and also to block the unoccupied site of the gold surface. The amount of antibody bound per nano-particle was then calculated based on protein assay results achieved from analysing the supernatant for unbound proteins using Bradford protein assay of Sigma, UK.

Immunosensor development

The carbon surface of the screen-printed electrode was directly used for the electro-deposition of polypyrrole-antibody-nano gold treatment. The freshly cleaned carbon was then covered with a 75 mM solution of polypyrrole plus antibody-nano gold and kept unstirred at room temperature. Chrono-amperometry measurements were carried out by setting 1.0V fix potential for 20 minutes onboard screen-printed Ag-AgCl pseudo-reference electrode. The antibody-nano gold coated electrode was then washed with 0.1M phosphate buffer to remove the excess of unbound antibody-nano gold. The layer is very stable if kept dry (under silica gel) for several weeks at 4 ^{o}C (Chaki and Vijayamohanan, 2002).

For tetracycline assays, various dilutions of tetracycline standard (0 – 250ppb) and tetracycline-HRP conjugate (20 µL) were added to the electrodes and incubated for 20-30 minutes at 37 ^{o}C. The electrode was washed 3 times with PBS-T and dried gently. The assay was then performed by adding 50 µl of TMB solution using chronoamperometry at 300mV for 100s - 200s. Calibration curve was fitted with a non-linear regression using 4-parameter logistic equation and the detection limit (LOD) was then calculated based on the following equation as described by Tijssen (1985):

$$LOD = \left(x \frac{a - d}{(a - d) - 3s} - 1 \right)^{-1/k}$$

where, s is for standard deviation of the zero value, a and d are the maximum and minimum values of calibration curve, x is the concentration at the EC_{50} value and k is the hill slope.

Sensor surface characterisation using Scanning Electron Microscopy

In this experiment, a XL30 SFEG scanning electron microscope (SEM) (FEI Company, Holland) was used to characterise the surface of bare and antibody-nano gold coated carbon surface screen printed electrode. The analysis was conducted based on the XLFEG/SFEG scanning electron microscope operating instruction manual.

Cross reactivity studies

Five epimer antibiotics in tetracycline group (tetracycline, oxytetracycline, chlortetracycline, demeclocycline and doxycycline) were used to examine the specificity of the immunosensor. The assays were conducted using various dilutions of four epimer tetracyclines (0 – 250ppb) and tetracycline-HRP conjugates (20 µL) were added to the electrodes and incubated for 20-30 minutes at 37 ^{o}C. The electrode was washed 3 times with PBS and dried gently. The assay was then performed by adding 50 µL of TMB solution using

chronoamperometry at 300mV for 100s - 200s. Cross reactivity with other antibiotic groups (streptomycine, vancomycine and chloremphenicol) also was conducted with similar procedure as above.

Comparative study between sensor and ELISA techniques

A set of triplicates of 12 chicken samples weighing 5 g each from various sources (commercial chicken from local outlet and supermarket, kampong chicken and MARDI experimental chicken) were mixed with 5 g sodium sulphate and placed in 50 mL centrifuge tube before grinding with a homogeniser. The overall procedure was summarized in Figure 2 below:

5 g sample + 5 g sodium sulphate + 25 ml mcllvain buffer (12.9 g citric acid monohydrate + 10.9 g $Na_2HPO_4.2H_2O$ + 37.2 g EDTA sodium salt, adjusted to pH 3.8 and top up to 1L H_2O)

- Homogenized (using rotary mixer) for 5-10 minutes
- Spinned at 4000 rpm, 10 minutes, 15°C

Supernatant (Step 1)

- Pelleted + 25 ml mcllvain buffer
- Homogenized for 5-10 minutes
- Spinned at 4000 rpm for 10 minutes, 15°C

Supernatant was pooled with supernatant from Step 1

- Filtered with Watman Filter Paper No. 4

Filtrated (was directly pipettes into the sensor strip and ELISA plate)

Figure 2: Extraction procedure for tetracycline analysis in meat samples using electrochemical sensor and ELISA kit.

Results and Discussion

The electrochemical immunosensor system developed in this work for tetracycline detection was based on direct competitive immunoassay format with HRP used as the enzyme label and TMB/H_2O_2 as the substrate/mediator system as illustrated in Figure 1. The higher the tetracycline in the sample, the lower the signal achieved from the electrochemical immunosensor.

Sensor surface characterisation before and after antibody immobilisation using Scanning Electron Microscopy (SEM)

Characterisation of carbon surface after electro-deposited with antibody-nano gold in polyprrole matrix was analysed by SEM analysis in comparison with the bare carbon electrode. The image of bare carbon electrode surface at 10,000x magnification showed a rough surface structure with the presence of non homogenous shape of carbon aggregate with an average size between 2 – 5 µm (Figure 2 a). From the observation of carbon electrode surface after electro- deposited with polypyrrole antibody nano-gold (Figure 2 b) the image surface showed slightly different (polypyrrole plus antibody nano-gold was coated on the carbon surface) when compared to the bare

structure. Thus, the SEM image analysis could visualise the changes of surface appearance after the attachment of antibody molecule on the carbon surface.

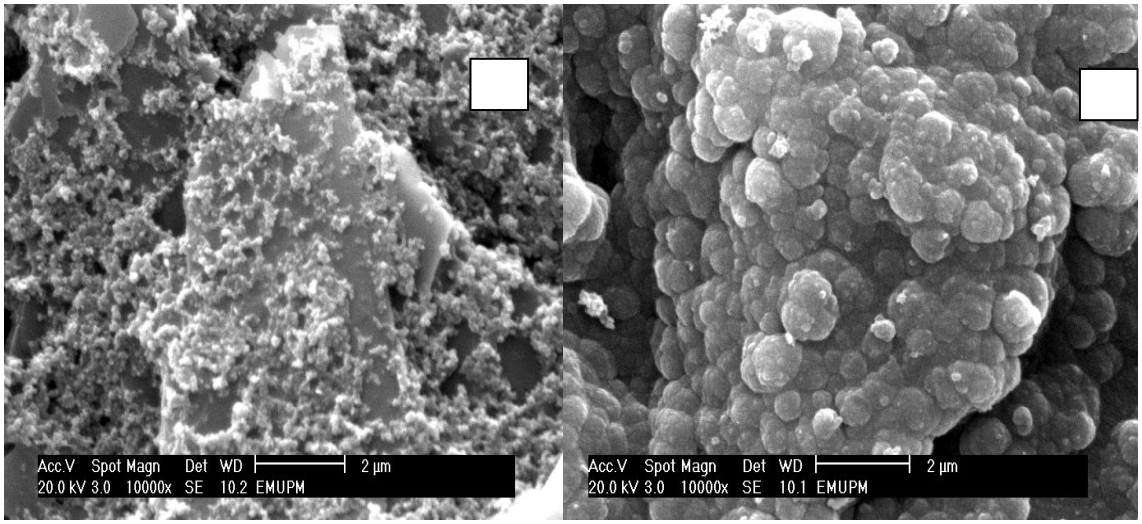

Figure 2. (a) Bare carbon electrode sensor surface under SEM analysis with 10,000x magnification and **(b)** After immobilization of Polypyrrole-nano gold-Anti Tetracycline antibody on sensor surface under SEM analysis with 10,000x magnification.

The immuno-sensor performance is influenced by the sensor surface morphology before and after attachment of antibody nano-gold layer. Microscopic techniques have become an important complementary tool for surface structure characterisation which provides information about the distribution of the immobilised molecule (Davis et al., 1998). Scanning Electron Microscope (SEM) has been used extensively for indirect characterisation of the presence of immobilised biological component on the electrode surface by visualisation of morphological changes on the surface appearance. For the determination of images at the molecular resolution, atomic force microscope has the ability to measure the intra-molecular force between transducer surface and bio-receptor (Davis et al., 1998). Therefore, to support the electrochemical data on the immuno-sensor performance, SEM analysis was conducted to examine the morphological changes on gold electrode surface before and after antibody immobilisation.

Tetracycline calibration curve using electro-deposited of polypyrrole nano gold anti-tetracycline antibody immuno- sensor

Standard calibration curve for tetracycline immuno-sensor was constructed based on direct competitive ELISA format. In this format, competitive binding of free tetracycline antibiotic (in sample) and conjugate tetracycline- HRP with immobilised antibody-nano gold on the carbon working electrode surface would form a direct format from which tetracycline could be quantified through the tetracycline-HRP conjugate. HRP activity was then determined electrochemically after the addition of 3,3',5,5'-tetramethylbenzidine-

hydrogen peroxide (TMB-H_2O_2) substrate. TMB has been reported to be suitable for use in ELISA with spectrophotometric measurement. It has been used as an electrochemical mediator and found to be a good substrate for electrochemical detection of low levels of HRP (Volpe *et al.*, 1998).

The assay was carried out with 10 µL of 1 mg mL^{-1} of anti-tetracycline antibody nano- gold electrodeposited on carbon working electrode. The electrode was washed with Phosphate Buffer (PB) before standard tetracycline (~99% purity, use as standard reference material) (0 – 250 ppb) and tetracycline- HRP conjugate was added (1 µg mL^{-1}, 20 µL) and incubated at room temperature (25 ºC) for 20-30 minutes. After washing with PBS-T, the TMB substrate was added to the sensor surface and the change in current was observed versus time for different tetracycline concentrations at a constant current of +300 mV. Figure 3 shows when tetracycline antibiotic concentration increased, the resulting signal was decreased (I, µA) which indicated that the decrease in current was inversely proportional to the tetracycline concentration. The current decreased linearly with the increase of tetracycline concentration in the range of 0- 250 ppb**.** Based on the calibration equation formula, the limit of detection of this printed SPCE coated antibody nano- gold sensor was calculated and was found to be at ~ 10 ppb.

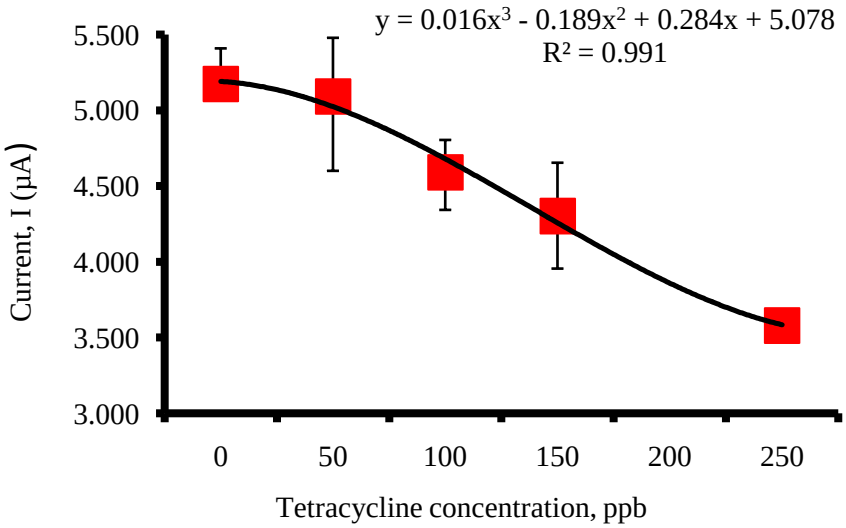

Figure 3. Tetracycline standard curve using competitive immunoassay on polypyrrole nano-gold anti-tetracycline antibody sensor

Sensor selectivity against other antibiotics in tetracycline family

Cross-reactivity describes the specificity of the antibodies and is an important analytical parameter regarding specificity and reliability of the immunosensor (Kawaguchi *et al*., 2007). Since antibodies can react with other substances present in the sample matrix which may lead to erroneous determination of the interest target. Therefore, the antibody specificity should be assessed prior to immunosensor

application to enable sample analyses without the need of a separation process.

The specificity of the sensor was investigated in relation to other antibiotics such as tetracyclines, oxytetracycline, chlortetracycline, demeclocycline and doxycycline as the most common antibiotic incorporated in poultry feed (Blackburn *et al*., 1994). The specificity of this tetracycline sensor with others in the tetracycline family was conducted by replacing tetracycline antibiotic with oxytetracycline, chlortetracycline, demeclocycline and doxycycline. The results showed that the tetracycline sensor was able to detect the other antibiotics belonging tetracycline family at almost ~ 100% reactivity (Figure 4).

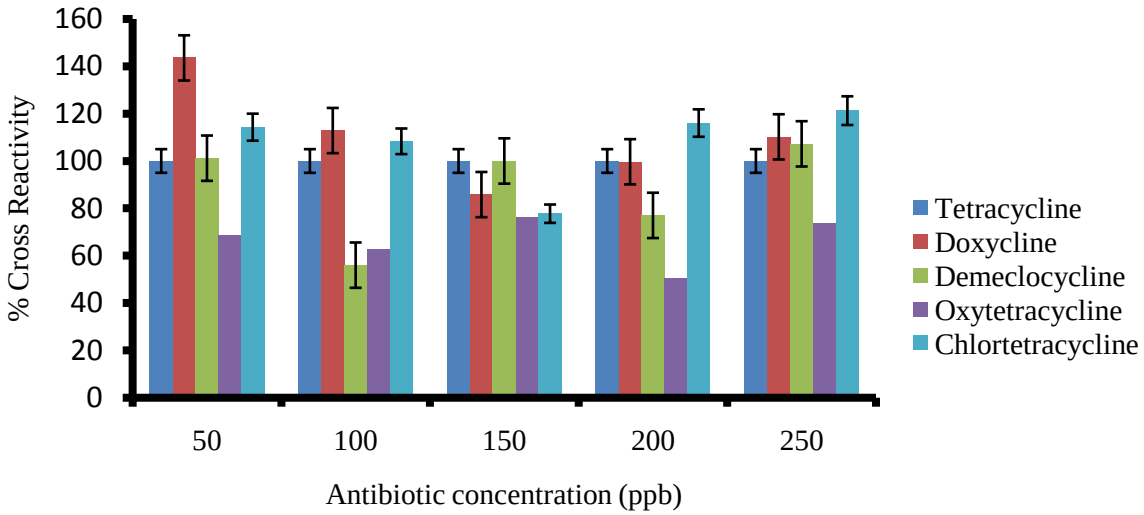

Figure 4. Sensor selectivity against others antibiotic in tetracycline family

Cross-reactivity of tetracycline sensor with the others in similar antibiotic group (streptomycine, vancomycine and chloramphenicol) also was conducted. All antibiotics tested showed slightly higher about ~ 20% cross- reactivity (Figure 5). This cross-reactivity of the tetracycline sensor could be due to the impurity of the polyclonal antibody used and also contamination during handling the antigen preparation in the laboratory.

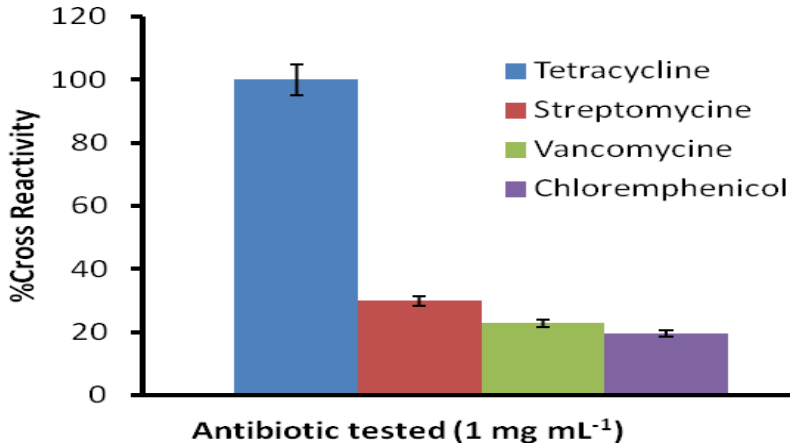

Figure 5. Sensor selectivity against others of the antibiotics family

Recovery rate analysis from the spiked free antibiotic chicken with tetracycline standard

The recovery values obtained were in agreement with the 2002/657/EC guidelines for validation method, where the acceptable trueness of measurements was assessed through recovery of additions of known amounts of the analytes to a blank matrix. In this study, antibiotic-free chicken was used as a blank matrix. Data corrected with the mean recovery were only acceptable when they fell within the following ranges; spiked antibiotic ≤ 1.0 µg kg^{-1} (≤ 1.0 ppb), the acceptable recovery was from 50 to 120%; spiked antibiotic > 1.0 to 10.0 µg kg^{-1} (>1.0 to 10.0 ppb) the acceptable recovery was from 70 to 110% and spiked antibiotic ≥10.0 µg kg^{-1} (≥ 10.0ppb) the acceptable recovery was from 80 to 110%.

Tetracycline recovery from spiked free antibiotic chicken with various tetracycline concentrations showed about 70 – 95% recovery (Figure 6). Therefore, according to the EC guidelines, the extraction method for tetracycline determination using the immuno-sensor is acceptable based on the percent recovery obtained and could be used for analysing the actual chicken samples or others meat samples.

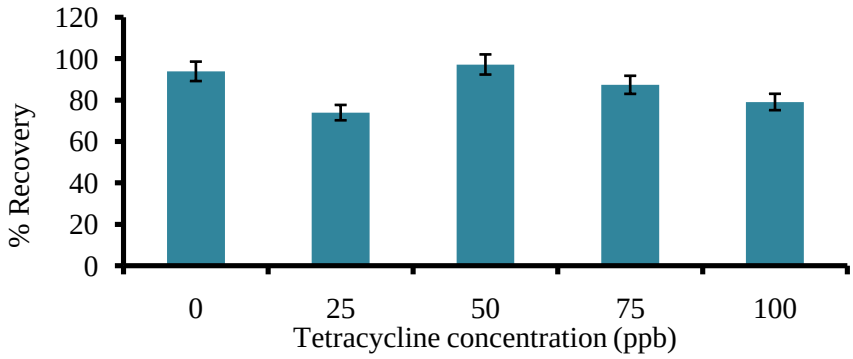

Figure 6. Recovery rate in artificially spiked tetracycline with various concentrations (ppb) (range 75-97%)

Comparison of immuno-sensors with commercial ELISA kit for tetracycline determination in chicken meat

The immuno-sensor is designed for rapid and sensitive detection of analytes in a single assay. The immuno-sensors based on electrochemical screen-printed electrode have been used for the detection of other chemicals such as aflatoxin (Tothill *et al*., 2011) with the detection limit in the range of 1-100ppb. However, conventional HPLC instrumentation method is the most sensitive method and remains the gold standard for detecting the presence of antibiotic and other chemical contamination in foods (USDA, 1995).

According to the Food and the Drug Administration (FDA) (USDA, 1995), immunological based methods represent the largest group of rapid test kits commercially available for food testing. The variety of commercially available immunological rapid test kits is well documented, but the most promising rapid checking of chemicals contamination is ELISA methods. According to RIDASCREEN ELISA test kit, the detection limit for most of the immunological based methods is approximately 6 ppb.

In this comparative study with the immunosensor method, commercial TETRACYCLINE ELISA kit from R-Biopharm, Germany was used. TETRACYCLINE ELISA kit was validated by collaborative studies within 27 laboratories and showed no significant difference ($P > 0.05$) were observed between batches with either visual or reader detection and is recommended for Official First Action Approval (Gangar *et al*., 1998).

Figure 7 shows significant correlation between the two detection methods (R^2 =0.9946) which demonstrated the comparable results obtained from both methods. The residue results also showed that chicken from local outlet sometimes contained higher tetracycline residues than the permissible residual level limit (100ppb) (Table 1). The developed sensor at the current setting compared very well to the detection range achieved by commercial immunochemical test kits which meets the sensitivity requirements set by the European Commission (EC regulation 123/2005).

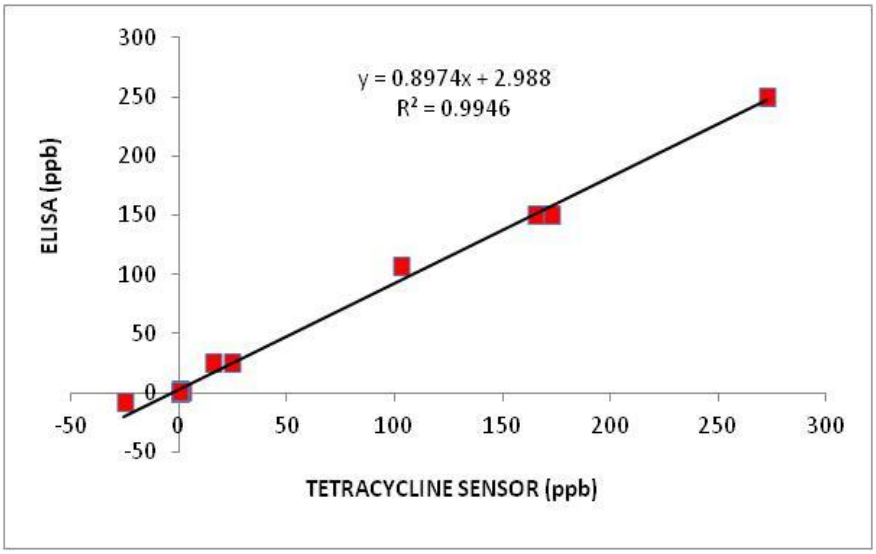

Figure 7: Relationship between immunosensor and ELISA methods for tetracycline analysis

Conclusion

A cost-effective and sensitive immunosensor for tetracycline antibiotic was fabricated using screen-printing technology as the sensor platform. The sensor was in-built with polypyrrole anti-tetracycline antibody-nano gold electro-coated on carbon working electrode. The developed immunosensor for tetracycline resulted in an improved detection limit when compared with an ELISA method. The level of detection using this sensor was equivalent to the lowest level of the permissible limit of tetracycline in meat set by the European Commission (100ppb or 100 μg L^{-1}). The developed electrochemical immunosensor using polypyrrole anti-tetracycline antibody-nano gold modified electrode has shown to be a useful device for screening and quantification of tetracycline antibiotic in meat samples for the livestock industry.

Table 1: Comparison of tetracycline level from local chicken sample analysis with Sensor and ELISA

Chicken samples	SENSOR (ppb)	ELISA (ppb)
AP2P2	1.94	0.71
AP2D2	0	0.74
AP3P2	0	0.78
AP3D2	0	0.44
AM1P2	24.40	25.00
AM2P2	0	0
AM2D2	0	0.55
AM3P2	0	1.08
AM3D2	0	0.44
AK1P2	0	2.97
AK1D2	165.40	150.00
AK2P2	0	0
AK3P2	15.67	26.00
AMR1D2	0	1.24
AZ3	172.67	150
RIC1	103.12	107.12
RIC3	272.83	250.00
AK2P2	-25.52	-7.70

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